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FIBROUS PROTEINS AND THEIR PRODUCTION

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This is a national phase filing of the Application No. PCT/DE99/02359, which was filed with the Patent Corporation Treaty on 3 August 1999, and is entitled to priority of the German Patent Application 198 34 909.2, filed 3 August 1998.

I. FIELD OF THE INVENTION

The present invention relates to a process for the production of fibrous proteins in plant cells, plant cells usable for this purpose and fibrous proteins obtained by the process.

II. BACKGROUND OF THE INVENTION

Fibrous proteins are proteins having mechanical stability, *e.g.*, resilience or elasticity. They form from precursor fibrous proteins which are polymerized and cross-linked, respectively. This requires the presence of repetitive amino acid sequences in the precursor fibrous proteins and the influence of proteins which process precursor fibrous proteins. Fibrous proteins are found in animal and human cells. Examples of fibrous proteins are collagen and elastin. Both are components of connective tissues, *e.g.*, skin, tendons, ligaments and blood vessels. Collagen forms by cross-linkage of tropocollagen molecules, while elastin is formed by cross-linkage of tropoelastin molecules.

Fibrous proteins are used for medical purposes and cosmetic purposes, respectively. To this end, they are frequently isolated from animal cells. This involves a great risk, since animal diseases, *e.g.*, BSE, can be transmitted to man in this way.

Therefore, it is the object of the present invention to provide a process by which fibrous proteins can be produced without the above risks.

According to the invention this is achieved by the subject matters defined in the claims.

III. SUMMARY OF THE INVENTION

The present invention relates to a process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

Furthermore, this invention concerns plant cells usable for this purpose and fibrous proteins obtained by this process.

IV. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to provide a process by which fibrous proteins can be produced without the above risks.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's findings that precursor fibrous proteins can be produced in plant cells, which can then be converted into the corresponding fibrous proteins by treatment with proteins processing them. In particular, he found that precursor fibrous proteins can be produced in both individual plant cells and plants. He also discovered that the conversion of precursor fibrous proteins into the corresponding fibrous proteins can be made *in vitro* and *in vivo*. In the latter case, this can be made, *e.g.*, in that the precursor fibrous protein is expressed in a plant cell together with the protein processing it. The applicant made his discoveries using individual plant cells and plants, particularly the potato plant.

According to the invention the applicant's findings are use for a process for the production of a fibrous protein, which comprises the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

The expression "fibrous protein" comprises a fibrous protein of any kind and origin. It may have two-dimensional or three-dimensional cross-linked structure. It can also be an animal or human fibrous protein. In addition, it may be available in wild-type or modified form. The latter comprises a fibrous protein whose amino acid sequence is modified as compared to the wild-type or modified form. The latter comprises a fibrous protein whose amino acid sequence is modified as compared to the wild-type sequence at one or more sites. Such modifications may be additions, substitutions, deletions and/or inversions of one or more amino acids. In particular, amino acids may be present which are preferably expressed in plant cells. Besides, the fibrous protein may be a fusion protein, the fusion partner being, *e.g.*, oleosin. This protein then enables the localization of the fibrous protein in the oil phase

of vegetable multiplication material. Fibrous proteins which are available in modified form have mechanical stability, *e.g.*, resilience or elasticity, which is at least comparable to that of the wild-type form. Preferred fibrous proteins are collagen and elastin as well as derivatives and fragments thereof, respectively. As regards a modified form, the above statements apply to them correspondingly.

The term "expression of a precursor fibrous protein" comprises any expression of a gene coding for a precursor fibrous protein in a plant cell, the precursor fibrous protein being convertible into the corresponding fibrous protein as usual, *e.g.*, by cross-linkage or polymerization. The above statements made on the expression "fibrous protein" apply here correspondingly. In addition, the precursor fibrous protein can be present with or without signal peptide. The former may be, *e.g.*, the natural or a foreign signal peptide, so that an extracellular localization of the precursor fibrous protein is enabled. In the latter, however, localization of the precursor fibrous protein is achieved in the cytoplasm. In addition, the precursor fibrous protein may have a control peptide so as to enable localization of the precursor fibrous protein in certain compartments of the plant cell, *e.g.*, ER, chloroplasts or vacuoles. Preferred precursor fibrous proteins are tropocollagen and tropoelastin as well as derivatives and fragments thereof, respectively. For the expression of a gene coding for a precursor fibrous protein it is possible to use conventional expression vectors for plant cells. They comprise regulatory elements, *e.g.*, enhancer, promoter and termination sequences detected in plant cells. Examples thereof are CaMV 35S promoter and termination sequences (Odell *et al.*, 1985, *Nature* 313:810-812). The expression vectors may also contain selection markers, *e.g.*, a neomycin or kanamycin resistance gene. In addition, the expression vectors may contain sequences which favor their introduction into plant cells. For example, the expression vectors may contain T-DNA of binary vectors, such as pSR 8-30 or pSR 8-35/1, when they shall be introduced into plants *via* Agrobacterium tumefaciens (Düring *et al.*, 1993, *Plant Journal* 3:587-598; Porsch *et al.*, 1998, *Plant Molecular Biology* 37:581-585). Besides, the expression vectors can also be introduced into plant cells by means of processes for which they do not require any special sequences. Such processes are, *e.g.*, microinjection, electroporation, DNA transfer by means of polyethylene glycol, liposome fusion or particle gun.

The expression "plant cell" comprises plant cells of any kind and origin. It may refer to individual plant cells, freshly isolated or established as a cell line, or those present in an

aggregation. The latter is, *e.g.*, a plant or part thereof. Examples of plants are monocotyl plants, such as corn, rice, wheat, barley and sugarcane, and dicotyl plants, such as potato, tobacco, tomato, tea, coffee, brassicaceaen, particularly rape and cabbage, and leguminaes, particularly pea, phaseolus, vicia and soybean.

The expression "protein processing precursor fibrous protein" comprises any protein which can convert a precursor fibrous protein into the corresponding fibrous protein. The conversion can be made as usual, *e.g.*, by cross-linkage or polymerization. Examples of such a protein are lysine oxidases. Also, proteinases may be concerned which, *e.g.*, in the case of collagen, have been described. The lysine oxidases and proteinases, respectively, may be present as such and as derivatives or fragments thereof, respectively. The above statements made on a modified form of a fibrous protein apply correspondingly to them.

The expression "incubation of a precursor fibrous protein with a protein processing it" comprises any incubation of these proteins by which the precursor fibrous protein can be converted into the corresponding fibrous protein. The incubation may be made, *e.g.*, *in vitro*. For this purpose, it is favorable to incubate the expressed precursor fibrous protein in solution with the protein processing it. The incubation can also be carried out *in vivo*. For this purpose, it is favorable to express not only the precursor fibrous protein but also the protein processing it in a plant cell. Both proteins can be expressed in different plant cells which are then combined whereby the precursor fibrous protein is incubated with the protein processing it. The precursor fibrous protein and the protein processing it can also be expressed in the same plant cell. Thus, both proteins are automatically incubated in this plant cell. The above statements made on the expression of a precursor fibrous protein apply correspondingly to the expression of a protein processing a precursor fibrous protein.

A further subject matter of the present invention relates to a plant cell which expresses a precursor fibrous protein and a protein processing it. Also, a plant cell is preferred which expresses only the latter of these proteins. Regarding the expressions "plant cell", "precursor fibrous protein" and "protein processing precursor fibrous protein" reference is made to the above statements. In addition, the plant cell may be available in the form of a multiplication material.

Common methods can be used for the production of a plant cell according to the invention. In supplement to the above statements, the production of a plant according to the invention which expresses a precursor fibrous protein, *e.g.*, tropoelastin, and a protein

processing it, *e.g.*, lysine oxidase, is described by way of example. In this connection, it is favorable to provide a cDNA coding for tropoelastin with CaNV 35S promoter and termination sequences and insert it in a binary vector, *e.g.*, pSR 8-30 and pSR 8-35/1, respectively. The same can be done with a cDNA coding for a lysine oxidase. The resulting DNA molecules are used for transforming bacteria, *e.g.*, *E. coli* S17-1 which are suitable for a transfer of the DNA molecules to *Agrobacterium tumefaciens, e.g.*, CV 3101. For this purpose, *E. coli* S17-1 and *Agrobacterium tumefaciens* GV 3101 are mixed with each other and incubated overnight. Agrobacteria which have taken up the DNA molecules are selected by growth on carbenicillin containing medium. They are then applied to cut-off potato plant leaves whose middle ribs were scratched several times and incubated in the dark for two days. Thereafter, the agrobacteria are removed and growth promoters are added to the potato plants, so that sprouts grow. They are cut off and used for cultivating new potato plants. The detection of the expression products tropoelastin and lysine oxidase and/or the resulting elastin is made by means of specific antibodies against these proteins. Reference is made to the below examples.

By means of the present invention it is possible to produce fibrous proteins in plant cells, particularly plants, in high purity. Therefore, the fibrous proteins are suitable for the most varying applications. They are found *e.g.*, in agriculture, chemistry, production of cosmetics and medicine. In the latter case, *e.g.*, the use of fibrous proteins for transplants and wound closures has to be mentioned. In particular, the fibrous proteins distinguish themselves in that they are free from animal or human viruses and pathogens, respectively. Moreover, the fibrous proteins can be produced in huge amounts. This applies particularly when they are isolated from plants cultivated in fields. Thus, the present invention represents a great contribution to providing pharmaceutical preparations safely and in great amounts.

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and

accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

V. EXAMPLES

A. Example 1: Production Of Elastin In Potato Plants

A cDNA is used for human elastin (Fazio, 1988, *Journal of Investigative Dermatology* 91:458-464). This cDNA is provided with an NcoI restriction site at its 5' end and with an XbaI restriction site at its 3' end by means of PCR. The resulting cDNA fragment is inserted in the vector pRT 100 which contains an expression cassette having CaNV 35S promoter and termination sequences (Töpfer *et al.*, 1987, *Nucleic Acids Research* 15:5890; Odell *et al.*, *supra*). Following cleavage using HindIII, the expression cassette containing the elastin cDNA is isolated and inserted in the binary vector pSR 8-30 (Düring *et al., supra*; Porsch *et al.*, *supra*). The expression vector pSR 8-30 elastin is obtained.

In addition, a cDNA for human lysine oxidase is used (Hämäläinen, 1991, *Genomics* 11:508-516). It is treated as described above and inserted in the binary vector pSR 8-30. The expression vector pSR 8-30 lysine oxidase is obtained.

The expression vectors pSR 8-30 elastin and pSR 8-30 lysine oxidase are used for transforming E. coli S17-1. The transformants are mixed with *Agrobacterium tumefaciens* CV 3101 and incubated at 27°C overnight (Koncz and Shell, 1986, *Molecular and General Genetics* 204:383-396; Koncz *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:131-135). Selection on carbenicillin is carried out, the bla gene necessary for this purpose being present in the above expression vectors. Selection clones of *Agrobacterium tumefaciens* are applied to cut-off leaves of potato plant cv. or named Désirée, whose middle ribs had been scratched several times and the plant is incubated in the dark at 20°C for 2 days. Thereafter, the agrobacteria are separated and growth promoters are added to the potato plant, so that sprouts form preferably. Moreover, non-transformed cells of the potato plant are killed by the addition of kanamycin to the plant medium. Rising sprouts are cut off and are allowed to grow roots on medium without plant growth substances but with kanamycin. The potato plants are further cultivated as usual.

The analysis of the expressed tropoelastin and lysine oxidase and/or the resulting elastin is achieved by antibodies in Western blot and ELISA, respectively, which are specific

to the individual proteins. For this purpose, whole protein or the intercellular wash liquid of the potato plant is isolated and used in the corresponding detection methods.

It shows that tropoelastin and lysine oxidase can be expressed in plant cells, particularly in a plant. Moreover, it shows that by the incubation of lysine oxidase with the tropoelastin the latter is converted into elastin which can be isolated in pure form.

B. Example 2: Production Of Collagen In Potato Plants

cDNAs are used which code for the subunits αl and α2 of human tropocollagen (Chu *et al.*, 1985, *Journal of Biological Chemistry* 260:2315-2320; Dickson *et al.*, 1985, *Nucleic Acids Res.* 13:3427-3438). Furthermore, cDNAs are used which code for human lysine oxidase, human procollagen C proteinase and procollagen N proteinase, respectively, from bovine animals (Hämäläinen *et al.*, *supra*; Li *et al.*, 1996, *Proc. Natl. Acad. Sci U.S.A.* 93:5127-5130; Colige *et al.*, 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94:2374-2379)

These DNAs are treated as described in Example 1 and inserted in the pSR 8-30 vector. The expression vectors pSR 8-30 tropocollagen α1, pSR 8-30 tropocollagen α2, pSR 8-30 lysine oxidase, pSR 8-30 C proteinase and pSR 8-30 N proteinase are obtained. The procedure is continued as described in Example 1.

It shows that tropocollagen and proteins processing it can be expressed in plant cells, particularly in a plant. In addition, it shows that collagen having a high degree of purity can be obtained.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.